

Applied Study

Comparing morphological and DNA-based bioassessment methodologies for macroinvertebrates in Neotropical streams: a case study from Ecuador

Gabriela Jijón^{1,2*}, Isabella M. Errigo^{1,2,3*}, Jessica Wicks¹, Natalie N. Goldston¹, Lillian Davis¹, Daniel Davis¹, Samantha Standring¹, John M. Chaston¹, Paul B. Frandsen^{1,4}, Blanca Rios-Touma²

¹ Department of Plant and Wildlife Sciences, Brigham Young University, Provo, UT 84602, USA

² Grupo de Investigación en Biodiversidad, Medio Ambiente y Salud (BIOMAS), Ingeniería Ambiental, Facultad de Ingenierías y Ciencias Aplicadas, Universidad de Las Américas, Quito CP 170503, Ecuador

³ Department of Natural Resources and the Environment, Cornell University, Ithaca, NY 14850, USA

⁴ Monte L Bean Life Science Museum, Brigham Young University, Provo, UT 84602, USA

Corresponding author: Blanca Rios-Touma (blanca.rios@udla.edu.ec); Paul B. Frandsen (paul_frandsen@byu.edu)



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Abstract

As unimpacted freshwater environments decline worldwide, developing effective and accurate biomonitoring approaches is critical for detecting ecosystem deterioration and facilitating conservation efforts. Freshwater macroinvertebrate communities comprise several phyla and thus a broad taxonomic expertise is required to identify these precisely. Molecular approaches for characterizing invertebrate communities have the potential to be more accurate, time-effective, and thus less costly than traditional morphological methods. However, evaluations comparing the two methods for macroinvertebrates are lacking in the Neotropics, where rapid deployment and increased accuracy are critical due to the escalating deterioration of freshwater environments. In this study, we compared the performance of DNA bulk tissue metabarcoding using an eDNA primer pair (fwhF2 & EPTDr2n) and morphological methodologies for identifying macroinvertebrate communities. Our aim was to evaluate the suitability of metabarcoding for assessing the biological quality of Ecuadorian streams along an altitudinal gradient. We found low taxonomic overlap between methods at family and genus ranks, though many molecular OTUs were unclassified at family and genus levels. Morphological identification resulted in higher Andean-Amazonian Biotic Index (AAMBI) scores for each site, with only three sites classified into the same biological category for both methodologies. One reason is that only family-level information is used for AAMBI. Existing DNA barcode reference libraries contained incomplete coverage of Ecuadorian taxa for specimens determined with the morphological approach, indicating a need to improve the representation of Ecuadorian taxa in these libraries to generate more accurate and complete metabarcoding determinations. Our study suggests that metabarcoding requires more development for freshwater biomonitoring in the Neotropics and should, at the moment, be used complementary to traditional identification approaches.

Key words: Andes, biomonitoring, freshwater invertebrates, metabarcoding, morphotaxonomy, Neotropics

* Equal contribution

Introduction

As the state of freshwater ecosystems declines worldwide, improved monitoring that informs effective management practices is essential to ensure their conservation (Revenga et al. 2005; Albert et al. 2020). Freshwater environments are threatened by various sources, including deforestation, agriculture, urbanization, mining, damming, and climate change (Azevedo-Santos et al. 2021; Antonelli 2022; Moi and Teixeira-de-Mello 2022). In the Neotropics, the condition of these ecosystems is declining at an alarming rate (Castello and Macedo 2016; Ríos-Touma and Ramírez 2019; Albert et al. 2020; Azevedo-Santos et al. 2021). This ongoing degradation highlights the importance of developing accurate and efficient monitoring methods to effectively assess and manage these ecosystems (Pelicice et al. 2017; Robinson et al. 2021). Previous monitoring efforts on neotropical streams have used bioindicators, including macroinvertebrates, fish, and microalgae, and developed indices to facilitate routine monitoring of freshwater ecosystem quality (Schulz and Martins-Junior 2001; Lobo et al. 2004; Baptista et al. 2007, Ríos-Touma et al. 2014; Ruiz-Picos et al. 2017). Macroinvertebrate taxa remain widely used as bioindicators because they are sensitive to habitat changes, ecologically diverse, easily sampled, and broadly distributed in freshwater habitats (Boothroyd and Stark 2000; Hodgkinson and Jackson 2005; Carter et al. 2006; Chang et al. 2014; Hauer and Resh 2017). These techniques conventionally rely on the morphological identification of collected specimens, which requires taxonomic expertise, a skill shown to be decreasing worldwide (Hopkins and Freckleton 2002; Wheeler et al. 2004). Moreover, these methods require high processing times and thus costs (Marshall et al. 2006; Pfrender et al. 2010; Hering et al. 2018; Blackman et al. 2019; Pereira-da-Conceicao et al. 2021; Chua et al. 2023). The morphological classification of larval specimens in the Neotropics is primarily performed at the genus level, limiting our understanding of the species present (Jackson et al. 2014).

Biomonitoring studies are increasingly carried out using molecular methods as they offer several advantages and complement traditional techniques (Duarte et al. 2021; Macher et al. 2024). The advantages include reducing cost and processing times, recovering a higher diversity (especially at the species level), relying less on the specialized expertise of taxonomists, and often, identifying rare taxa (Hajibabaei et al. 2019; Duarte et al. 2021). However, in the Neotropics, metabarcoding techniques have been applied primarily to vertebrates (Milan et al. 2020; Zinger et al. 2020). To date, no study has evaluated the use of metabarcoding to amplify DNA from freshwater benthic macroinvertebrates in the Neotropics. Previous efforts comparing conventional freshwater benthic macroinvertebrate morpho-taxonomic approaches have primarily taken place in the Global North (Elbrecht et al. 2017; Emilson et al. 2017; Fernández et al. 2018; Serrana et al. 2019; Duarte et al. 2021). These studies have shown that molecular approaches can result in higher sensitivity (Emilson et al. 2017), reduced sampling and processing time (Nichols et al. 2020), and lower cost (Aylagas et al. 2018) compared to conventional methods of identification (Thomsen and Willerslev 2015). Despite the advantages of molecular techniques in specimen characterization, there are drawbacks to

adopting DNA-based identification that must be addressed before widespread adoption. Limitations of molecular methods include challenges in determining abundance from sequence data, primer bias, and reference database incompleteness (Pawlowski et al. 2018; Schenekar et al. 2020; Marques et al. 2021). The underrepresentation of Neotropical taxa in DNA reference databases potentially hinders organism determination and highlights the need for additional studies that characterize the extent of this issue to justify increased investment in generating reference databases in the region (Zinger et al. 2020).

In this study, we collected standard replicate freshwater macroinvertebrate samples over an elevational gradient in eastern Ecuador to compare the efficiency of both morpho-taxonomic and DNA bulk-tissue metabarcoding methodologies. Our main objective was to compare both identification methods and evaluate the suitability of DNA metabarcoding to substitute the ongoing morphological monitoring efforts in the country. This is the first study to apply and test the accuracy of DNA-based freshwater benthic macroinvertebrate identification approaches in the Neotropics.

Materials and methods

Study site

We sampled streams and rivers in Ecuador's eastern Andean mountain range, located within the Napo (100,518 km²) and Pastaza (39,504 km²) river basins in the upper part of the Amazon river basin. Ten sites were sampled along an altitudinal gradient ranging from 210 to 2838 m.a.s.l. (Fig. 1, Suppl. material 1: table S1), comprising the Sucumbios, Pastaza, and Tungurahua provinces in September 2021.

Macroinvertebrate sampling

For each site, we collected two samples; one sample was to be used for metabarcoding analysis and the other for morphological taxonomic identification. We sampled using a D-net (mesh size: 250 µm) by moving upstream for three minutes and collecting in all the habitats available in the sampling reach, including sweeping stream edge vegetation (Acosta et al. 2009). This technique has been shown to work well for stream quality assessment (Bradley and Ormerod 2002) and has successfully been used in previous studies in the region (e.g., Rios-Touma et al. 2022). Samples were collected sequentially by the same person, with the second sample collected in a reach immediately upstream of the first sample. Large debris, including rocks and aquatic plants, were sampled by dislodging the organisms by hand into the net. We placed the samples in Whirlpak bags and preserved them with denatured 95% ethanol. Upon returning from the field, the samples were thoroughly sieved (mesh size: 250 µm) to remove sharp debris and large sediment and stored at 4 °C. For each site, one sample was identified with morpho-taxonomic methods and the other was identified with DNA metabarcoding. The morphological samples were processed at Universidad de Las Américas in Quito, Ecuador, and the metabarcoding samples were processed at Brigham Young University in Utah, USA.

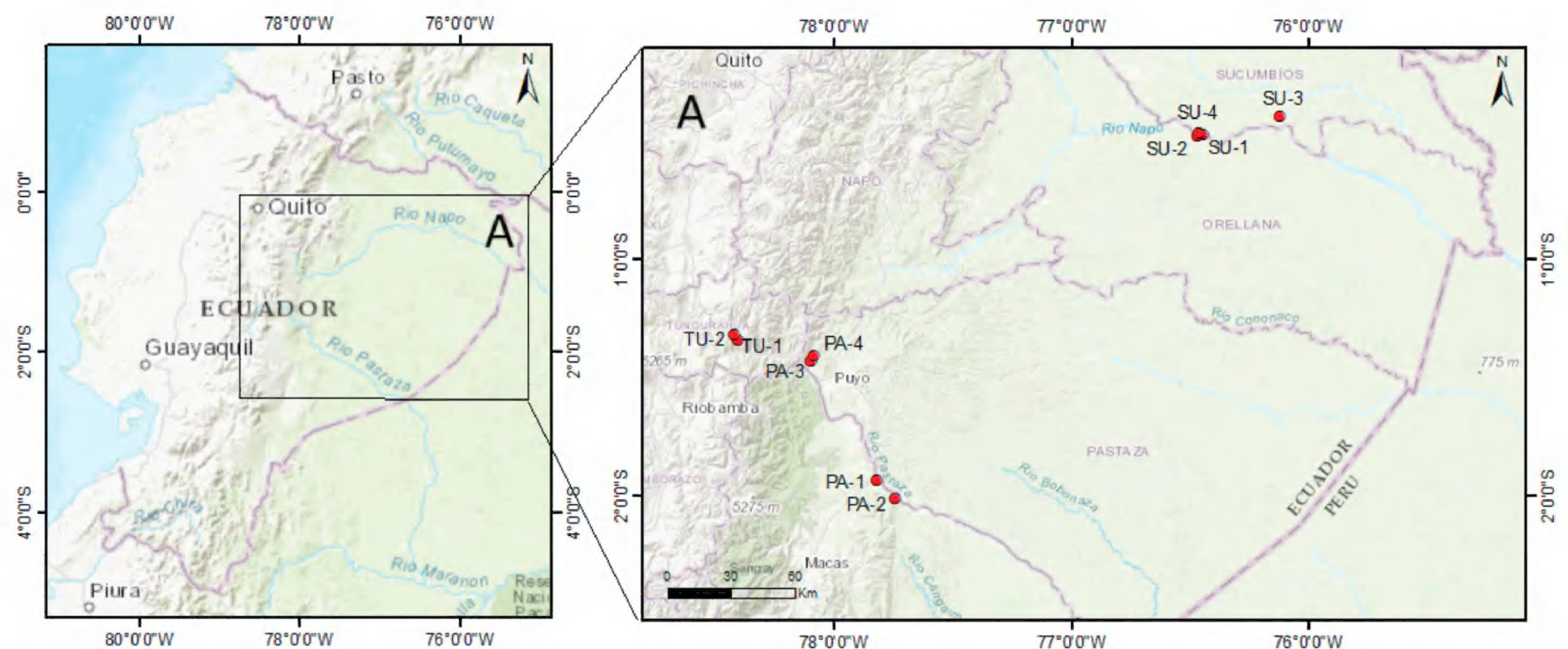


Figure 1. Map of the ten study sites in Ecuador's Andean and Amazonian provinces of Sucumbios, Pastaza, and Tungurahua.

Laboratory procedures

Morphological classification

All macroinvertebrate specimens ($> 250 \mu\text{m}$) from the kick samples were analyzed for morpho-taxonomic classification. The specimens were sorted from the debris and identified under an Olympus SZ61 stereo microscope (Olympus, Tokyo, Japan). Macroinvertebrate specimens were classified using keys from Domínguez and Fernández (2009) and Hamada et al. (2018).

Tissue homogenization

We followed the protocols described in Buchner et al. (2021) to process the metabarcoding samples with a few modifications. We manually cleaned the samples by removing rocks while retaining fine sediment and organic material (Pereira-da-Conceicao et al. 2021). Following Elbrecht et al. (2017), we removed large insects and placed a 4 mm portion of each insect back in the sample to avoid overrepresenting large insect DNA. We used the Homgeek Professional 10-Speed Blender (model number NY-8608MCA) to blend all metabarcoding samples.

Before homogenizing each sample, we sterilized the blender with a custom decontamination solution that included bleach, NaOH, Alconox, and NaHCO_3 (Buchner et al. 2021). We blended 250 mL of the cleaning solution on power level 5 out of 10 for 20 seconds. We then rinsed the blender by blending 300 mL of milli-Q, ultra-pure water for 30 seconds.

To homogenize samples, we placed the cleaned sample in the blender. If the sample was not large enough to cover the blender blades, we poured ethanol into the blender until the blades were fully covered. We then blended the samples for three minutes at power level 7 out of 10. We poured the homogenized sample back into the Whirl-pak bag where it was initially stored and removed excess ethanol from the sample, leaving enough ethanol to cover the homogenized sample. We followed the same blending protocols on two negative controls with 250 mL of milli-Q, ultra-pure water. One control was run halfway through processing the samples (after the 5th sample), and one was run at the end.

DNA extraction and quantification

Before DNA extraction, we homogenized the sample by vortexing for 30 seconds, transferred 2 mL of the sample to a microcentrifuge tube, and then used a ThermoSavant SpeedVac 1010 (Thermo Fisher Scientific, Waltham, MA, USA) to spin the samples at room temperature until all the ethanol evaporated (usually about 40 minutes but varied by sample). We placed 50 mg of the dried sample directly into the Zymo Research BashingBead Lysis Tubes.

We extracted DNA from each sample (including the two tissue homogenization controls from the bulk-tissue homogenization step) using the ZymoBIOMICS Miniprep Kit (D4300) following manufacturer protocols with the following modifications. After step one, samples were incubated in Lysis Buffer for 10 minutes at 65 °C. Samples were then further homogenized with a Geno/Grinder 2010 (SPEX SamplePrep, Metuchen, NJ, USA) for 3 min at 1250 rcf. In step 11, during the DNA elution, we transferred 50 µL of ZymoBIOMICS DNase/RNase Free Water to the column, incubated for 7 minutes at room temperature, then centrifuged using the parameters specified in the protocol. The eluate was added back to the column and incubated at room temperature for 7 minutes before centrifuging in two subsequent steps to increase total DNA recovery. Subsequently, DNA was quantified using a Qubit 3 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA; Suppl. material 1: table S2). Extractions were stored in a -80 °C freezer until sequencing.

PCR amplification

We carried out a two-step PCR amplification process using the freshwater macroinvertebrate degenerate primer sets (fwhF2/EPTDr2n) developed by Leese et al. (2021) for eDNA studies. Our step one PCR reaction contained 25 µL Apex BioResearch Products Hot Start *Taq* (Product number 42-143) Master Mix, 3 µL of template DNA, and 2 µL of primer. We ran the amplification on a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) with the following program: 95 °C for 15 minutes; 10 cycles of 95 °C for 30 seconds, 55 °C (decreasing by 1 °C each cycle) for 1 minute and 30 seconds, then 72 °C for 1 minute and 30 seconds; 25 cycles of 95 °C for 30 seconds, 55 °C for 1 minute and 30 seconds, and 72 °C for 1 minute and 30 seconds; then a final extension of 68 °C for 10 minutes. During the second PCR step, sample-specific indexes and Illumina adapters were added to the amplified DNA, as instructed in Leese et al. (2021). To do this, we used 25 µL of Apex BioResearch Products Hot Start *Taq* (Product number 42-143) Master Mix, 2 µL of primer, and 1 µL of amplified template DNA from reaction one. Step two was run on the same thermocycler instruments with the following program: 95 °C for 15 minutes; 18 cycles of 95 °C for 30 seconds and 72 °C for 2 minutes, with a final extension of 68 °C for 10 minutes. In addition to amplifying our extracted homogenization controls, we ran one PCR control during PCR amplification using 30 µL of milli-Q, ultra-pure water. PCR amplifications were visualized with 1% agarose gel electrophoresis.

We did not conduct PCR or extraction replicates. However, future studies should consider the inclusion of replicates, which may aid in identifying rare taxa and reduce biases (Shirazi et al. 2021; Bohmann et al. 2022). Due to

the cost and time associated with taxonomically identifying all individual macroinvertebrates in a sample, collecting a single kick sample at each site is a common and widely accepted practice in biomonitoring work (Bradley and Ormerod 2002).

Library prep/cleanup/normalization

We used the Just-A-Plate Normalization Plate Kit (CharmBiotech) to normalize samples following the manufacturer's instructions, scaling down the volumes to match the size of the PCR reaction. We then pooled 10 µL of each sample together by plate.

Pooled libraries were evaluated on an Agilent FemtoPulse (Agilent Technologies, Santa Clara, CA, USA) to estimate fragment size distributions. Size selection was then carried out on a Sage Science Blue Pippin (Sage Science, Beverly, MA, USA), where samples were selected for 250–500 bps. We estimated the molarity of the final sample with qPCR on a Thermo Scientific ViiA 7 (Thermo Fisher Scientific, Waltham, MA, USA). The fragment analysis, size selection, and qPCR were all performed by the Brigham Young University DNA Sequencing Center.

Sequencing

Along with samples from three other projects, samples for this project were sequenced on an Illumina MiSeq (Illumina, San Diego, CA, USA) over three sequencing cartridges (two nano and one standard cartridge) using v2 chemistry, 2×250 bp sequencing, and following the manufacturer's instructions. At the end of the sequencing run, the samples were automatically demultiplexed using the unique sample-associated barcodes delineated on the mapper file uploaded to the MiSeq instrument prior to sequencing.

Data analysis

Sequence Processing

We performed all initial sequence processing using QIIME 2 (q2) 2022.2 (Bolyen et al. 2019). Raw sequence data from each sample were first trimmed to remove the indexes, adapters, and primer sequences and then denoised with the DADA2 denoise-paired function (via q2-dada2; Callahan et al. 2016). We clustered the sequences at 98% to generate molecular operational taxonomic units (mOTUs) and then exported the representative sequences file. An alpha rarefaction curve was created by plotting the mOTUs against the sequencing depth to assess whether the sequencing depth was adequate to capture the samples' diversity (Hall and Beiko 2018). We generated this using 100 iterative samples at each sequencing depth (Suppl. material 1: fig. S2).

Taxonomic designation

To assign taxonomic information to mOTUs, we used a three-step framework to ensure correct classifications. As a first step, we used BLAST 2.13.0+ (Altschul et al. 1990) to search each mOTU against the GenBank nucleotide database.

We then filtered the hits by length and percent identity, keeping only the longest hits with the highest percent identity to assign the single most likely taxonomic match to the sequence. After filtering the hits, we kept species assignments with identity > 97%, genus assignments with identity > 94%, and family assignments with identity > 91%. With the taxonomic assignments, we created an mOTU table in QIIME 2 using the “feature-table summarize” command and added taxonomic information from the BLAST search to the corresponding records in the mOTU table. In the second step, because simple BLAST percent identity cutoffs may not result in correct identifications, we generated neighbor-joining trees within BOLD Systems (Ratnasingham and Hebert 2007) to verify the classification of each mOTU. If the phylogenetic tree indicated that the representative sequence was nested within a clade with barcodes of the same taxonomic rank, the determination was assigned to that particular rank. Lastly, to test whether a different classification approach may yield additional classified sequences, we used COIClassifier (Porter and Hajibabaei 2018) and kept classifications that were made with scores > 0.93 (order) or > 0.95 (family, genus, and species). For downstream analysis, within each site, we collapsed mOTUs that were assigned to the same taxonomic rank.

For the morphology-based classification, we identified each individual insect to genus except for immature larvae, damaged specimens, and insect families that lacked genus-level keys or required additional processing to make taxonomic identification possible (e.g., Ceratopogonidae, Chironomidae, Culicidae, Empididae, Ephydriidae, Limnichidae, Limoniidae, Scirtidae, Tabanidae, and Tipulidae). Non-insect specimens were identified to the lowest taxonomic level possible based on the literature, developmental stage, and specimen condition (Suppl. material 1: table S5). Round and hair worms were identified to phylum, segmented worms to subclass, and flatworms to order. Cnidarians were determined to class and mollusks to either family or genus. Within non-insect arthropods, springtails were classified to subclass and mites to order. Within crustaceans, ostracods and copepods were classified to class, branchiopods to order and malacostracan to genus. Pupae and terrestrial macroinvertebrates were not morphologically identified.

To evaluate the status of the DNA barcoding database for Ecuador, we determined if the families and genera in our morphological identifications were represented by DNA barcodes from the country in the sequence databases. To do this, we searched for the family and genus names on BOLD (<https://www.boldsystems.org/>) and GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) in October 2023 and recorded the presence or absence of COI sequences in Ecuador for each taxon.

Biotic index calculation

An important aspect of determining the quality of a stream in the Andes is to assign each site a score in the Andean-Amazonian Biotic Index (AAMBI; Encalada et al. 2019), which was developed based on Ríos-Touma et al. (2014) and Roldán-Pérez (2016) and assigns pollution tolerance scores to each family from 1 to 10. The pollution tolerance scores for the families present are added to calculate the index score at each site. An AAMBI score of >121 indicates “excellent,” 90–120 “very good,” 50–89 “good,” 36–49 “fair,” and <35 “bad” aquatic

biological integrity. To assess whether the bulk-tissue metabarcoding approach produced comparable biological quality index scores to the morpho-taxonomic approach, we calculated the AAMBI score for each method for each site using the classified mOTUs and morphologically determined families.

Results

Molecular-based identification

The bulk-tissue samples produced 127,707 paired-end reads for the ten samples analyzed in our study. Following sequence trimming, we retained 119,320 paired-end reads (see Suppl. material 1: table S3 for total and counts per site). Two of our negative controls (one DNA extraction negative control and the PCR negative control) did not have an amplification band on the agarose gel (Suppl. material 1: fig. S1) and yielded no reads. The second DNA extraction control yielded 451 reads that were all assigned to *Drosophila melanogaster*, a common lab contaminant. We identified terrestrial taxa at five sites (i.e., SU-3, SU-4, PA-1, PA-3, PA-4). These taxa were removed before performing richness counts and calculating biological indices to maintain a fair comparison with the aquatic taxa identified using morphological methods.

We clustered sequences into 341 mOTUs, 339 of which we could classify to at least the class level (Suppl. material 1: supplementary item S5). Of those, 328 mOTUs were classified to order (96%), 282 to family (83%), 88 to genus (26%), and only 22 to species (6%). Overall, we recovered 37 different families, 40 different genera, and 22 different species across sites for the molecular-based samples. The number of families, genera, and species identified varied widely across sites (Suppl. material 1: tables S4, S5). For example, SU-1 and PA-1 were the most rich at the family level, with 12 and 13 families identified. However, PA-3 was the richest at the genus level, with mOTUs classified into 11 genera. Most sites had very few mOTUs successfully assigned to species; the site with the most mOTUs assigned at the species level was site SU-1, with six species-assigned mOTUs, followed by TU-2, with four. SU-2 had the fewest mOTUs assigned to specific taxa, with a single identified family and no genus or species-level determinations. The rarefaction curve comparing sequencing depth to the number of observed mOTUs approached an asymptote; however, a higher sequencing depth (>100,000 reads per sample) would likely capture more unique and rare taxa (Suppl. material 1: fig. S2).

Morphological-based identification

The morphologically identified samples were classified from phylum to genus and included 50 different families and 60 different genera across the sites. Notably, of these, 26 families and 40 genera had no representative sequences from Ecuador in the BOLD or GenBank databases, including commonly found families in morphological samples, such as Ceratopogonidae, Empididae, and Limoniidae (Suppl. material 1: tables S5, S6). Additionally, several common genera had no deposited barcodes for Ecuador, including *Heterelmis*, *Tenagobia*, *Eupera*, and *Psephenus* (Suppl. material 1: tables S5, S6). In contrast to the

results from metabarcoding, TU-2 yielded the highest number of morphologically identified families, with 20, and PA-1 yielded the highest number of genera, with 25. SU-2 had the lowest number of families and genera, with two identified families and two identified genera (Suppl. material 1: tables S4, S5).

Comparison between methodologies

The morphological-based method produced more unique taxon determinations per site than the metabarcoding method. The only exceptions were PA-3 and TU-1, which had an equal number of families to the morphological approach (Fig. 2), and SU-3, in which more genera were classified with metabarcoding than with morphology (Suppl. material 1: fig. S3, table S4). However, at nearly every site, there were many mOTUs that were unclassified at both the family (Fig. 2) and genus (Suppl. material 1: fig. S3) levels.

At the family rank, there was overlapping identification of macroinvertebrates at all sites, ranging from one (SU-2 and PA-2) to eight families (SU-1 and TU-2; Fig. 3). Chironomidae was the only family that overlapped between both methods at all sites. Baetidae and Elmidae overlapped between both methods at half of the sites, while Ceratopogonidae, Leptohyphidae, and Simuliidae overlapped between the methodologies at three sites. The morphological and molecular approaches identified at least two genera at the same site for half of the sites. The overlap ranged from zero (SU-2 to 4 and PA-1 to 2) to four taxa (TU-2) at the genus level across the sites (Suppl. material 1: fig. S4), with *Leptohyphes* and *Simulium* being the most frequent overlapping genera between approaches. Notably, 17% and 74% of the total mOTUs were not classified to the family and genus ranks, respectively, likely contributing to the reduced overlap between methodologies (Fig. 2, Suppl. material 1: fig. S3).

Biological index

The estimated AAMBI biological categories were the same across methods for only three sites, SU-2, and PA-2, PA-3 (Fig. 4). For all other sites, the AAMBI biological category estimated via DNA metabarcoding was lower than the category estimated with morphological identification, although, across all sites, the two methods were correlated (Suppl. material 1: table S6, fig. S5; Pearson's $r = 0.88$, $p = 0.00077$ and Spearman's $\rho = 0.82$, $p = 0.0037$). The first sample from Pastaza Province (PA-1) and the second sample from Tungurahua Province (TU-2) had the highest average AAMBI biological scores across both methods (Fig. 4, Suppl. material 1: table S7). Two sites, PA-1 (AAMBI = 114) and TU-2 (AAMBI = 119) were classified as "very good" from morphological determinations (Suppl. material 1: table S7, fig. S5), while the highest biological category estimated from DNA-based taxon counts was "good" for the PA-1 and PA-3 (AAMBI = 52 and 56, respectively) sites. PA-2 (AAMBI Metabarcoding = 14 and Morphological = 26) and SU-2 (AAMBI Metabarcoding = 2 and Morphological = 7) received the lowest biological category score of "poor" with both methods, with SU-2 having the lowest overall scores (Fig. 4, Suppl. material 1: table S7). These two sites were sampled in the largest order streams of the study, the Napo (SU-2) and Pastaza Rivers (PA-2).

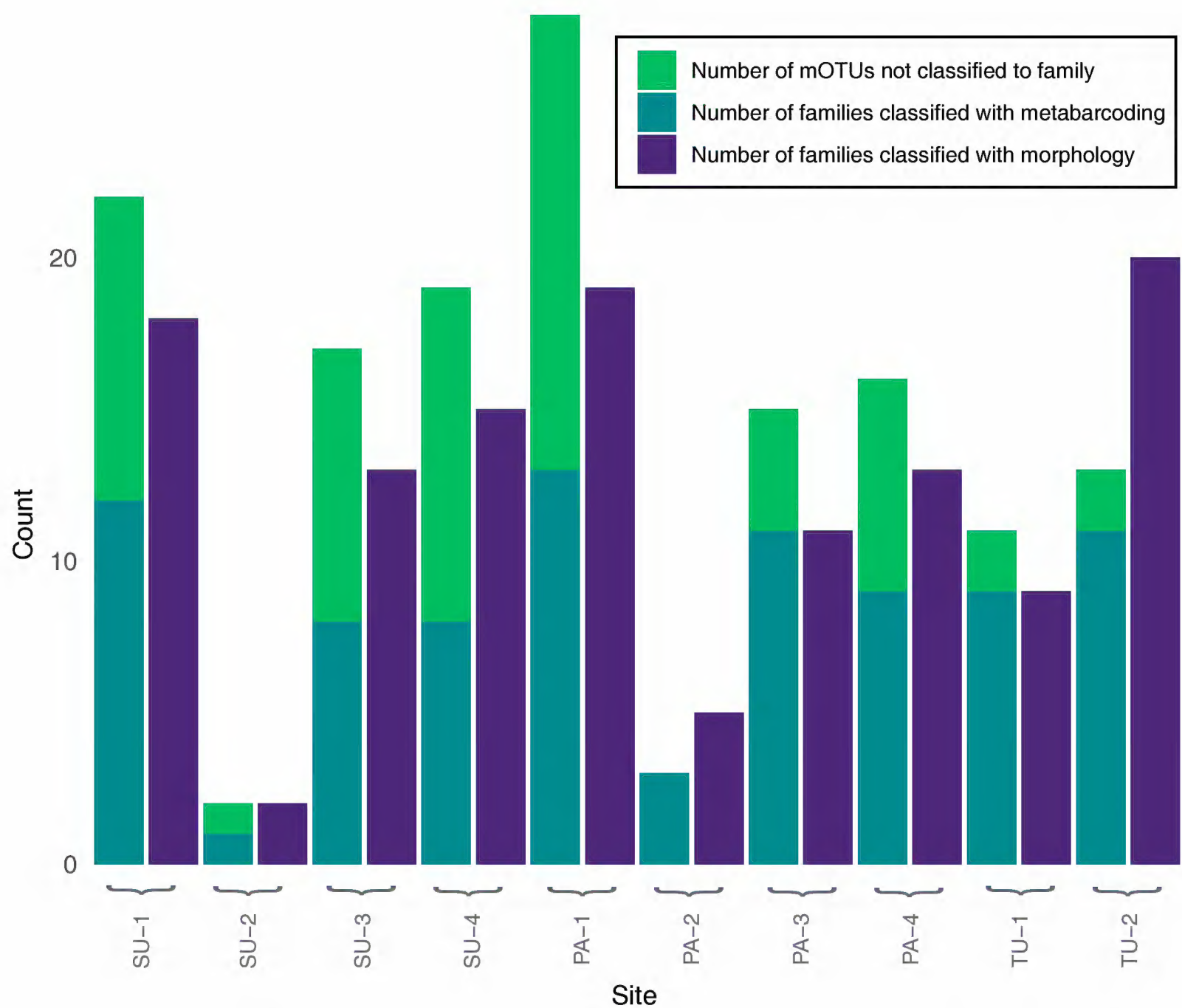


Figure 2. Unique family counts for morphological and molecular methods. The number of individual mOTUs that were not classified at the family level are also shown stacked on top of the metabarcoding family count, likely representing unidentified diversity.

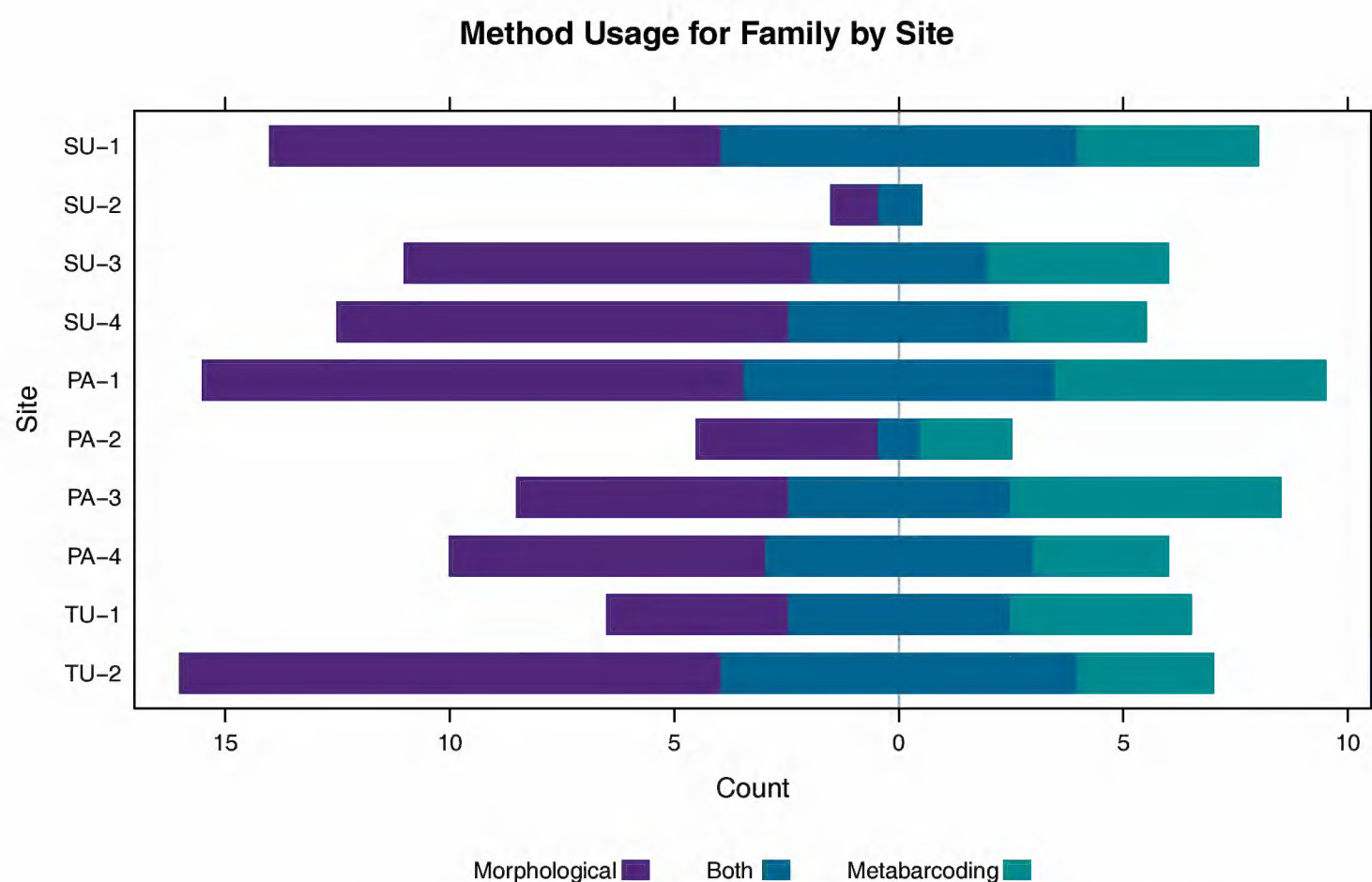


Figure 3. Horizontal bar plot of overlaps in families classified between morphological and DNA-based approaches at a family rank across the ten collection sites in Ecuador. Non-overlapping family counts for the morphological-based method (purple) and molecular-based method (green), and overlapping family counts between the methods (blue).



Figure 4. AAMBI scores and biological categories of the site-method combinations across the ten sites. The AAMBI index score's biological quality categories: >121 "excellent" (blue), 90–120 "very good" (green), 50–89 "good" (yellow), 36–49 "fair" (orange), and <35 "poor" (red).

Discussion

Our study assessed the performance of a DNA metabarcoding-based identification method against the classical morpho-taxonomic approach for characterizing benthic communities in Ecuadorian streams with the aim of determining whether a bulk tissue metabarcoding approach is currently applicable for biomonitoring programs in the region. This study represents a first step in understanding the baseline requirements and challenges for deploying a relatively low-cost and time-effective DNA-based identification approach in a region where molecular techniques have not been widely applied in aquatic macroinvertebrate studies (De León et al. 2020).

We characterized macroinvertebrates from sites ranging from low-elevation Amazonian rivers to high-elevation Andean streams. Regardless of the determination approach, chironomid midges were found at all sites. In agreement with previous studies, we identified changes in subfamily composition across the elevational gradient, with Chironominae found more commonly at lower elevations and Orthocladiinae found more frequently at higher elevations (Acosta and Prat 2010; Villamarín et al. 2021). Our results also coincided with previous morphological studies in the high-elevation Andes, with both methods successfully identifying genera common to high-elevation streams, e.g., the mayfly (Ephemeroptera) genera *Andesiops* and *Leptohyphes* and the black fly (Diptera) genus *Simulium* (Vimos-Lojano et al. 2020). The site with the highest taxonomic richness for both methods and one of the highest average AAMBI scores was from a sample collected in a small stream tributary to Río Pastaza.

Sites with the lowest richness and AAMBI scores from both methods were from samples collected from the largest rivers in the study, Río Napo and Río Pastaza. The reduced diversity observed in these samples might be attributed to challenges in benthic sample collection in high-order streams where samples can be collected only in shallow margins, possibly limiting the stream's full benthic diversity collection (Ramírez 2010).

Although most comparative studies have found that DNA-based techniques produce higher richness estimates than morphology-based techniques (Elbrecht et al. 2017; Hajibabaei et al. 2019; Serrana et al. 2019; Pereira-da-Conceicao et al. 2021; Keck et al. 2022b), we found the reverse, with morphology-based ID's producing higher richness and higher AAMBI scores for most sites. However, a considerable number of mOTUs were not identified to family and/or genus, suggesting our results are at least partly biased due to incomplete reference databases. This highlights the need to populate barcode databases with local barcodes to make the application of molecular methods more successful in the future. Currently, the authors of the present study are working towards populating Ecuadorian freshwater macroinvertebrate taxa in reference libraries.

Primer bias also likely contributed to the underestimation of richness and the low overlap between methods (Elbrecht et al. 2017; Hajibabaei et al. 2019; Serrana et al. 2019; Gleason et al. 2021; Keck et al. 2022a, 2022b; Larano et al. 2023). For example, the primers that we used have previously been reported to be biased against the aquatic insect order Trichoptera, Turbellaria, and several Crustacea (Leese et al. 2021; Brantschen et al. 2022), and in PA-1, PA-3, TU-1 and TU-2 sites, we identified families of Trichoptera with morphology that were not represented in the metabarcoding data. Using nonspecific primers that are more suitable for eDNA samples than bulk tissue samples may have hindered taxonomic resolution. This could be ameliorated through using primers that are more appropriate for bulk samples (e.g., fwhF2 and fwhR2; Vamos et al. 2017) or the use of multiple primer sets (Gleason et al. 2021; Keck et al. 2022a).

Additionally, differences between taxa recovered with morphological and molecular sampling from the same site could be reduced by planning for collection, extraction, and PCR replicates along with deeper sequencing in the budget and study design. Given the heterogeneity of the stream microhabitats and invertebrate community distributions within a stream, not all diversity is captured in a single benthic sample, and variation in replicate samples is likely to be present (Furse et al. 1981; Bush et al. 2019). Sampling replicates could help to address heterogeneity within sites. Although our rarefaction curves approached an asymptote, saturation in mOTU numbers might not have been necessarily obtained. Increased sequencing depth (>100,000 reads per sample) is likely necessary to recover the full site diversity (particularly rare taxa) (Shirazi et al. 2021).

Despite the generally lower levels of taxon detection with metabarcoding, we were able to classify some taxa into genus or species ranks that are difficult or impossible to identify morphologically and revealed higher genus-level richness in groups like Diptera (Suppl. material 1: table S5). Morphology-based identifications were biased against certain groups, e.g., chironomids and culicids, which were mostly identified to subfamily and tribe ranks because they required further processing or lacked the keys to make genus-level determinations (Lizarralde de Grosso 2009; Paggi 2009; Corrêa and Gil-Azevedo 2018; da Silva et al. 2018; Fusari et al. 2018). Additionally, earlier instars and damaged specimens were

challenging to identify morphologically, a problem that molecular methods do not encounter (Domínguez and Fernández 2009; Curry et al. 2018; Hamada et al. 2018). However, the improved species-level resolution is not used in the generation of AAMBI scores, which rely on family level data, and may be responsible for the incongruence in the biotic index scores between methods.

Without a complete reference library, DNA-based identification can result in weak matches with species from other regions, underestimation of diversity, and erroneous community characterizations (Venter and Bezuidenhout 2016; Sales et al. 2021). The lack of an adequate reference library for freshwater invertebrate taxa is widespread across the Neotropics, hampering the application of DNA-based approaches (Hadi et al. 2016; De León et al. 2020; Zinger et al. 2020; Sales et al. 2021; Bukowski et al. 2022). Freshwater taxa are widely used as bioindicators, and metabarcoding could provide a more efficient and effective method of assessing freshwater invertebrate diversity, especially because these ecosystems are facing rapid changes due to multiple stressors (Ríos-Touma and Ramírez 2019). To this end, molecular methods show great promise in Neotropical biomonitoring, but concentrated efforts must first be made to complete reference databases and improve primers before they can be effectively implemented on a large scale.

Inadequate DNA barcode reference databases are not isolated to the Neotropics and have been reported globally. Previous assessments of reference barcode databases have found incomplete databases for aquatic invertebrates in regions like North America, Europe, and China (Curry et al. 2018; Weigand et al. 2019; Li et al. 2022). Some aquatic macroinvertebrate groups are especially underrepresented, for example, the insect orders Diptera, Ephemeroptera, Odonata, and Hemiptera, and non-insect groups such as flatworms, annelids, mollusks, arachnids, and nematodes (Curry et al. 2018; Weigand et al. 2019; Li et al. 2022). Thus, future efforts must be devoted to equitably populating libraries with local sequences to improve DNA-based identification accuracy for aquatic taxa. Improved collaboration between experienced taxonomists and geneticists to identify and sequence reference species is essential for making higher taxonomic resolution possible for metabarcoding projects. This is especially important in the Neotropics, where much of the biodiversity has not yet been recorded, and habitat degradation is of high concern (Venter and Bezuidenhout 2016; Ríos-Touma and Ramírez 2019; de Santana et al. 2021).

In addition to improving reference libraries, other aspects of DNA-based identification approaches must be improved to increase their accuracy and utility as biomonitoring tools. Biomonitoring studies would also benefit from abundance estimates obtained from DNA-based identification approaches, which require methodology improvements and tools such as amplification bias correction, improved modeling techniques, and/or extraction of population genetics information, for example, that could allow for more accurate taxa quantification (Andres et al. 2023; Shelton et al. 2023).

Our study suggests that molecular methods cannot replace morphology-based identification methods for Neotropical macroinvertebrate biomonitoring and emphasizes an integrated approach of DNA-based identification and traditional methods, as this can provide a more detailed characterization of community composition and stream diversity (Keck et al. 2022b). As taxonomic resolution varies in morphological identifications due to reliance on available

keys, DNA-based identification methods improve the identification of groups that are difficult to determine morphologically or do not have complete keys for the region (Elbrecht et al. 2017; Pereira-da-Conceicao et al. 2021; Keck et al. 2022a). Molecular identification is also advantageous for characterizing cryptic species and immature larvae and is generally more easily applied in taxonomic re-identification than morphological approaches (Gill et al. 2016; Venter and Bezuidenhout 2016; Curry et al. 2018; De León et al. 2020).

Conclusion

Our study compared the traditional morphological identification method with DNA metabarcoding to characterize macroinvertebrate communities across stream sites along an altitudinal gradient to determine whether molecular-based methods can currently be implemented effectively in Ecuador and regionally. It is the first comparative study of its kind in the Neotropics, and studies like this are essential to assess the application feasibility of new methods in the region. The morphological approach identified more taxa and estimated a higher overall richness (when only considering those identified taxa) than the DNA-based approach. There was a low taxonomic overlap between the methods at family and genus ranks. The lack of overlap between methods illustrated the differing capabilities of each approach in identifying different invertebrate groups. Incomplete reference libraries for Ecuadorian taxa influenced the reduced number of families, genera, and species classified with the DNA-based method. Other factors, such as using primers that are more suitable for eDNA studies than for bulk samples, lack of replicates, and a shallow sequencing depth, also likely contributed to these compositional discrepancies between approaches and should be adjusted in future studies. However, despite the incomplete taxonomic identification of families with metabarcoding, the biological index scores calculated for both methods were significantly correlated, indicating a promising future for using molecular methods in biomonitoring efforts. Our findings indicate that DNA-based identification methods can complement traditional identification approaches but cannot, at present, replace the morphological-based approach in biomonitoring studies in the Neotropical region. To improve the application of molecular-based approaches, increased investment in improving DNA barcode reference libraries and reducing bias in PCR primers is critical.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: BRT, PBF. Data curation: BRT, GJ, IME, PBF. Formal analysis: NNG, JW, GJ, JMC, BRT, SS, DD, LD, IME, PBF. Funding acquisition: PBF, BRT. Investigation: BRT, JMC, IME, PBF, GJ. Methodology: BRT, PBF, IME, JMC. Project administration: IME, GJ, BRT, PBF. Resources: BRT. Supervision: BRT, JMC, PBF. Validation: GJ, IME, BRT, PBF. Visualization: PBF, GJ, IME, BRT, NNG, SS. Writing – original draft: PBF, GJ, IME, BRT. Writing – review and editing: DD, IME, SS, NNG, GJ, JMC, BRT, JW, LD, PBF.

Author ORCIDs

Daniel Davis  <https://orcid.org/0009-0009-3461-1807>

Paul B. Frandsen  <https://orcid.org/0000-0002-4801-7579>

Blanca Rios-Touma  <https://orcid.org/0000-0002-3921-0908>

Data availability

Raw sequencing reads are available on the NCBI SRA under BioProject PRJNA1209988, including BioSample accessions SAMN46235618-SAMN46235627 (Suppl. material 1: table S1). Representative sequences, mOTU table, raw reads, UNIX commands, and R scripts are available on FigShare, <https://doi.org/10.6084/m9.figshare.c.7619435>.

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Supplementary material 1

Supplementary data

Authors: Gabriela Jijón, Isabella M. Errigo, Jessica Wicks, Natalie N. Goldston, Lillian Davis, Daniel Davis, Samantha Standring, John M. Chaston, Paul B. Frandsen, Blanca Rios-Touma

Data type: pdf

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